

**Research Article**

## MOLECULAR DETECTION, ISOLATION, AND PATHOLOGY OF BOVINE TUBERCULOSIS IN AN ORGANIZED FARM IN ASSAM, INDIA

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**ABSTRACT:** Bovine tuberculosis (bTB) is a well-known zoonotic disease that affects cattle all over the world and results in significant economic loss, particularly in impoverished nations. The present communication describes the pathology, isolation and molecular detection of *Mycobacterium* in an organized farm of Assam which has the previous records on animals with confirmed *M. bovis* infection. During the period 2020-2021, a total of 40 animals (4 males and 36 females) of one year and above were included in the present study for screening of bovine tuberculosis by single intradermal comparative tuberculin test (SICCT). The milk, nasal swabs were collected from only tuberculin positive cattle and the tissue samples from necropsied animal and then processed for bacteriology, histopathology and molecular detection from direct samples. Out of 40, four cows showed positive reactor by SICCT and out of these four, one animal died. At necropsy, there was presence of circumscribed yellowish white lesions of various sizes and numbers. The smear prepared from granulomatous tissue samples showed the presence of acid-fast bacilli by Ziehl-Neelsen stain. *Mycobacterium* could be isolated from tissue samples. The DNA extracted from the samples could amplify *Mycobacteria* genus specific hsp65 gene and MTBC specific a 123-bp segment of the insertion sequence IS6110.

**Key words:** Acid fast, Bovine, Histopathology, IS6110-PCR, SICCT, Tuberculosis.

### INTRODUCTION

*Mycobacterium bovis* causes bovine tuberculosis (bTB), a chronic, infectious, and progressive illness of cattle, other domesticated animals, and some free or confined wildlife species. It is characterized by the formation of non-vascular granulomas known as tubercles which occur most frequently in lungs, lymph nodes, liver, intestine and kidney. The bovine TB is a significant veterinary disease that can spread to human mainly through ingestion of unpasteurized milk and rarely through inhaling infective droplets (Torgerson and Torgerson 2008). However congenital and suckling colostrum and milk from infected animals are also the route of infection which are widely described by World Organization for Animal Health (OIE 2009). Bovine

tuberculosis is still a major problem in developing countries for public health, food safety, wildlife, and the economy of livestock industries. It is also a major zoonotic disease mainly occurring amongst workers of dairy farms, veterinarians, slaughterhouse workers and consumers who consume contaminated raw milk and other products. Clinically the disease is characterized by debilitated condition, cough, decreasing milk production, labored breathing etc (Srivastava *et al.* 2008). It is an OIE listed disease which occurs worldwide and interfere seriously with international trade of animals and animal products (Roex *et al.* 2015).

Tuberculin skin test (TST) has traditionally been used to determine the prevalence of infection in human and animals by using the purified protein derivatives (PPD)

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of *Mycobacterium bovis* which is fastidious organism and requires 8-12 weeks of incubation to grow in solid media for isolation (Drobniewski *et al.* 2003, Lambert *et al.* 2006). In the field, diagnosis of bovine Tuberculosis is based on clinical and postmortem alterations observed by the veterinarians, which may be confused with some other diseases like aspergillosis, aspiration pneumonia, pleurisy and contagious bovine pleuropneumonia (CBPP) etc. Now a days, molecular techniques like polymerase chain reaction (PCR) can detect Mycobacterial DNA in the isolates obtained from clinical/tissue samples thus representing a valid additional tool for the ante-mortem and postmortem diagnosis of bovine Tuberculosis (Monteros *et al.* 1998). The present communication describes the pathology, isolation and molecular detection of *Mycobacterium* in an organized farm of Assam.

## MATERIALS AND METHODS

The study was conducted in an organized cattle farm at Karmrup (M) district of Assam, India. This organized farm has the previous records on animals with confirmed *M. bovis* infection. A 6-year-old cow was suffering from chronic respiratory illness from 2-3 months. So that a total of 40 animals present in the farm (4 males and 36 females) of 1 year and above were included in the present study for the screening of bovine tuberculosis.

### Single intradermal comparative cervical tuberculin (SICCT) test

Intradermal tuberculin test is the only suitable method for the diagnosis of bovine tuberculosis (bTB) in live animals in field condition. So, a single intradermal comparative tuberculin test (SICCT) was performed. This test compares immune responses to *M. bovis* (bovine) and *M. avium* (avian) tuberculin in the cervical region.

All the animals present in the farm were subjected to SICCT test as per the guidelines from the World Organization for Animal Health (OIE 2018). Briefly, the test was carried out in the middle third of the neck of each animal where avian tuberculin PPD-2500 (PPD-A; Avian Tuberculin PPD from culture of *Mycobacterium avium* subsp. Avium, strain D4ER) [Prionics Lelystad BV, Lelystad, The Netherlands] and bovine tuberculin PPD-3000 antigens (PPD-B; Bovine Tuberculin PPD from culture of *Mycobacterium bovis*, strain AN5) [Prionics Lelystad BV, Lelystad, The Netherlands] were injected (*i.e.*, 0.1 ml of PPD) in two sites of neck 12 cm apart. Skin thicknesses were measured with caliper before and 72 h after PPD injections. The animals were considered to be positive if the difference of the skin thickness at the injection sites is 5 mm or above and if the difference of the skin thickness is less than 2 or 3mm then it was considered as negative.

### Collection of the sample

The milk, nasal swabs were collected from only tuberculin positive cattle and the tissue samples (inflamed or caseous) were collected from the carcass that suspected to be died due to bovine tuberculosis. The samples were transferred carefully to sterile screw-top plastic container accordingly (Himedia). The milk samples (approx. 40 ml) were collected from the infected cattle and for histopathological examination, tissue samples were collected in 10% neutral buffered formalin. All the samples carried out immediately at 4°C to the laboratory of Division of Animal Health, ICAR Research Complex, NEH Umiam, Meghalaya-793103, India and processed under Class-III Biosafety cabinet (ESCO, Singapore). All the sampling procedure followed by the guidelines of OIE (OIE 2013).



Fig. 1. The tuberculin positive cow.

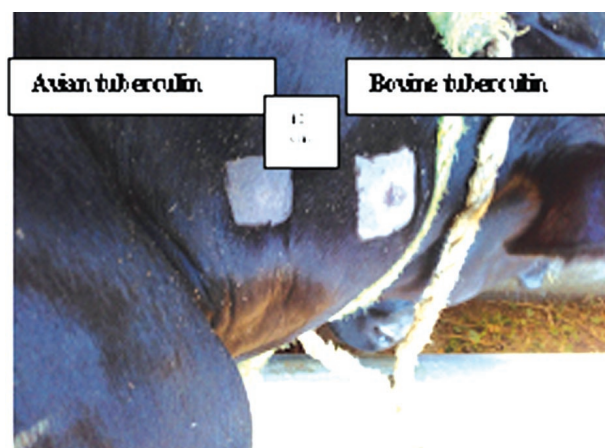


Fig. 2. The comparative tuberculin test.

### Gross pathology

The necropsy examination for the detection of lesions of bovine tuberculosis was thoroughly identified and examined in all tissues. A tentative diagnosis of bovine tuberculosis can be made following the finding of typical lesions during necropsy. At necropsy, gross alterations in different organs were properly recorded by taking all aseptic precautions in association with the collection of tissues for bacteriological and the associated laboratory examination. The aim of aseptic collection is to minimize the human risk and prevent contamination of tissues with soil and faces (Corner 1994).

### Histopathological Examination

For histopathological examination, tissue samples extracted from the dead cows were fixed in 10% buffered formalin. Paraffin blocks prepared with routine laboratory methods and the samples were then sectioned at 5- $\mu$ m thickness and were stained with routine hematoxylin and eosin (H&E) and Ziehl–Neelsen (ZN) methods and were examined by light microscope (Tuncay and Hatipoglu 2018).

### Detection of *Mycobacterium* spp.

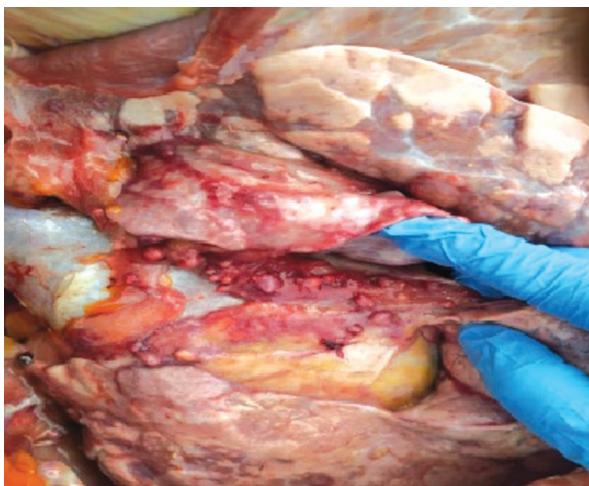
The collected milk, nasal swab and tissue samples were processed for both bacteriology and molecular detection (PCR). To process the specimens for culture and smear preparation, the tissue was first homogenized using a mortar and pestle, followed by decontamination with an alkali (2–4% sodium hydroxide) for 10–15 minutes at room temperature and then neutralized and the homogenized mixture was taken in a sterile 15 ml centrifuge tube with a screw cap. The suspension is

centrifuged at 3000 rpm for 15 minutes, the supernatant discarded, and the sediment or pellet is used for culture and microscopic examination (Lambert *et al.* 2006). The milk samples were centrifuged at 15000 rpm for 12 mins and washed three times with 35–40 ml of PBS. The pellet was suspended in 0.1 ml of PBS. About 0.5 ml of specimen was inoculated into both pyruvate and glycerol-based Lowenstein-Jensen medium and cultured at 37°C and observed once weekly for 8–12 weeks (Drobniewski *et al.* 2003). For evidence of *Mycobacterium*, *Mycobacteria* genus specific Hsp65 (Telenti *et al.* 1993) and *Mycobacterium* Tuberculosis Complex (MTBC) specific IS6110 based PCR (Eisenach *et al.* 1990) were employed on the direct tissue samples after decontamination.

### Identification of the bacteria by smear preparation

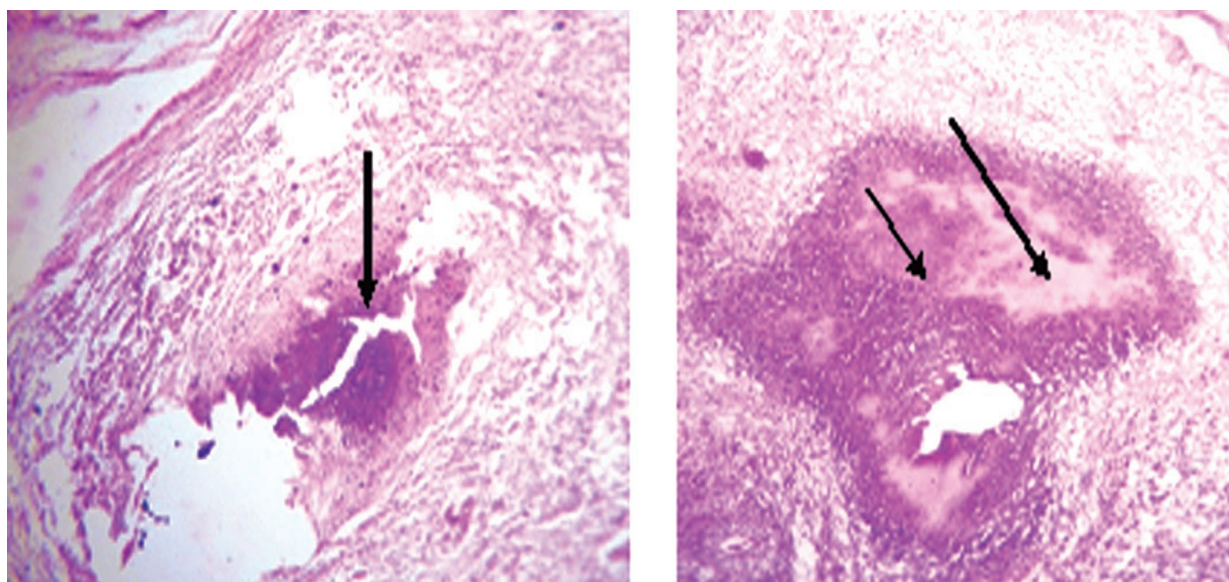
Acid Fastness/Ziehl-Neelsen Staining Technique (“Hot” Z-N technique) was carried out on the direct tissues to detect acid fast bacilli from granulomatous tissue at room temperature using standard protocol mentioned by Sa’idu *et al.* (2015).

Direct smears were prepared from tissues presenting tuberculous lesions. The collected tissues were first crushed using a sterile pestle and mortar and homogenized using a stomacher then placed on a clean and labelled grease-free glass slide. The smeared slides were air-dried and heat-fixed by passing them gently over the flame (Bunsen burner). The smears were then stained with carbol fuchsin and heated the stain until vapor just begins to rise (*i.e.* about 60°C) and allowed to stand for 5 minutes. After that the smear was washed with tap water (to remove excess stain from the slide). The slide was decolorized



**Fig. 3 and Fig. 4. Lung of affected cow showing tubercle nodules (left) and caseotic necrosis (right).**





**Fig. 5. Histopathological findings of tubercle nodule.**

[The granuloma was consists of a central area of caseous necrosis with variable calcification, surrounded by epithelioid cells, Langhan's giant cells (arrows), lymphocytes, with a fibrous capsule infiltrated by lymphocytes (H&EX200)].

with 3% acid alcohol for 1 min. After decolorization, the slide was counterstained with methylene blue for 1 minute and then washed and allowed to air-dry. Finally, the slide was viewed in an optical microscope under 100 X (an oil emersion objective). Acid fast bacilli appeared red, straight or slightly curved rod occurring singly or in a cluster indicates the presence of tubercle bacilli.

### **Molecular Detection of Bovine tuberculosis DNA extraction and quantification**

DNA was extracted from the milk, nasal swab and tissue samples via the Qiagen DNA extraction kit-based method (DNeasy® Blood & Tissue, Qiagen, USA) as per manufacturer protocol. The isolated DNA was used for molecular process. The DNA quantification and assessment was done by NanoDrop (Thermo Scientific).

### **Direct PCR-based detection from clinical samples**

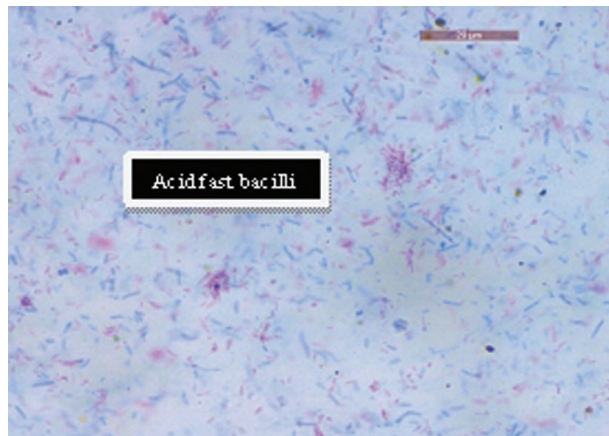
The amplification of DNA extracted from samples was carried out for the detection of *Mycobacterium* genus specific hsp65 gene and *Mycobacterium tuberculosis* complex (MTBC) specific insertion sequence IS6110. The target gene hsp65 was amplified using following primers: hsp65 F'-ACCAACGATGGTGTGTCCAT and R'-CTTGTCGAACCGCATACCCT (Telenti *et al.* 1993). The PCR mixture (total 25 µl) was as follows: A total of 12.5 µl master mix which containing (distilled water, dNTPS mix, reaction buffer solution, and MgCl<sub>2</sub>), 1 µl of forward and 1µl of reverse primers (10 pmol), 5 µl of

DNA and 5.5µl of nuclease free water. The mixture was transferred to a Thermal Cycler and subjected to the following condition: 95°C/4 min, 35 cycles of 95°C/30 sec, 59°C/30sec, 72°C/35 sec and final extension at 72°C/ 7 min.

The MTBC specific PCR was performed on a total volume of 25µL as per the procedure mentioned by Eisenach *et al.* (1990). The detection of MTBC DNA by IS6110-PCR by amplifying a 123-bp segment of the insertion sequence IS6110 using following primer: IS6110F'-CCTGCGAGCGTAGGCGTCGG and IS6110R'-CTCGTCCAGCGCCGCTTCGG. Cycle conditions were as follows: 95° C for 5 min, 35 cycles of amplification (95°C for 30 s followed by 70°C for 35 s and 72°C for 30 s) and 72°C for 5 min. 5 µl of the amplified product was subjected to electrophoresis on a 2% (w/v) agarose gel with 0.03% of ethidium bromide. *M. bovis* AN5 strain was used as a positive control. After electrophoresis DNA was visualized and recorded by Gel documentation (Cleaver Scientific, U.K). The samples that were TB positive were further analyzed to determine the specific mycobacterium strain.

### **RESULTS AND DISCUSSION**

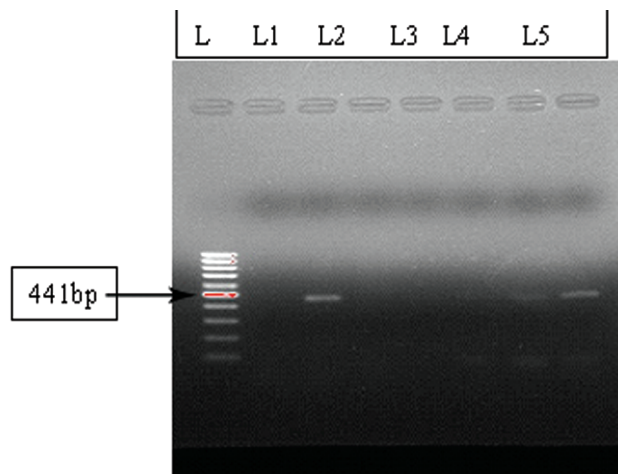
The results of the Single intradermal comparative tuberculin test (SICTT) have been expressed following the guidelines provided by the OIE, (2018). Out of the total forty (40) tested animals, four (10.00%) were positive by SICTT (Fig. 1). However, milk and nasal



**Fig. 6. Z-N Staining of acid-fast bacilli.**

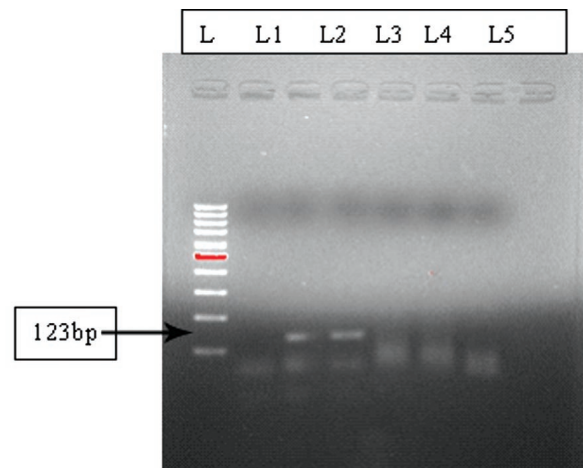


**Fig. 7. Culture growth on LJ media.**



**Fig. 8. hsp65 PCR showing positive result for tubercle nodule and pus sample from lung.**

[(Lane - 100bp DNA ladder, L1-negative control, L2-positive control (*M bovis* AN5), L6 & L7-positive tubercle sample)].



**Fig. 9. IS6110 showing positive band in positive control and the tubercle nodule sample.**

[(Lane - 100bp DNA ladder, L1-negative control, L2-positive tubercle sample & L3-positive control)].

smear from the SICCT positive animals could not demonstrate the *Mycobacterium* organism by Ziehl-Neelsen stain. The intradermal tuberculin test is the OIE recommended test for screening against bTB which detects cell responses of T helper type1 (Th1) lymphocytes (OIE 2009, Schiller *et al.* 2010). Almost similar prevalence rate (14.31%) was reported by Thakur *et al.* (2010) from Himachal Pradesh. However, higher positivity (28%) was recorded by Konch *et al.* (2018) from Assam and (28.8%) by Bassessar *et al.* (2014) from Jabalpur. Stages of infection, energy and immune system of individual animal can be responsible for sensitivity and specificity of tuberculin test in different animals and areas. The chronic nature of the tested animals in the present study might be resulted in higher prevalence rate.

Nahar *et al.* (2011) also could not able to detect acid-fast bacilli in impression smear from nasal swab and illustrated that the diseased cattle rarely shed the organism at detection levels in nasal discharge.

Out of these four SICCT positive cow, one animal aged eight (8) years showed clinical signs like inappetence, sneezing, frequent fever, pale mucus membrane, slight swelling of pre-scapular lymph-node and emaciation (Fig. 2). The cow died and post mortem examination was performed. At necropsy, the carcass was highly emaciated with the presence of circumscribed yellowish white lesions of various sizes and numbers. Macroscopically at postmortem, the most common changes seen in the lungs were presence of yellowish white tubercular lesions of various sizes and numbers (Fig. 3). Apart from lungs,



similar encapsulated nodules with varying diameter could be observed in liver also. On incision, yellowish white caseous exudate comes out from the nodules (Fig. 4). Marked consolidation with collapse of lung parenchyma was also prominent. Almost similar gross alterations were reported by Jhones *et al.* (1997), Kumar and Swamy (2005), and Konch *et al.* (2018). Corner (1994) reported that up to 95% visible tuberculous lesions could be identified by examination of the lung and associated lymph nodes in cattle. Involvement of these lymph nodes might be due to transmission of the disease via respiratory route.

Microscopically, the nodular lesion was characterized by central areas of caseous necrosis, round, oval or irregular and often coalescing, eosinophilic, homogenous, with necrotic debris (Fig. 5). Sometimes the necrotic area was surrounded by epithelioid cells, Langhans-type multinucleated giant cells, plasma cells and a collar of lymphocytes. The granulomatous lesion was surrounded by a layer of fibrous connective tissue (Fig. 6). Similar lesions were also seen in liver and lymph nodes. Similar lesions were described earlier (Cassidy *et al.* 1999, Nahar *et al.* 2011, Goswami *et al.* 2014).

The smear prepared from granulomatous tissue samples showed the presence of acid fast bacilli (Fig. 7) by Ziehl-Neelsen stain. The organism could be demonstrated both in the central caseo-necrotic mass as well as in granulomatous tissue. Konch *et al.* (2017) also demonstrated acid fast bacilli in the nasal smear of tuberculin positive animals. Goswami *et al.* (2014) demonstrated in lung tissue and Prakash *et al.* (2015) demonstrated both in the lung as well as mediastinal lymph nodes.

During isolation of *Mycobacterium spp.* in Lowenstein-Jensen both pyruvate and glycerol-based media, no growth could be observed in milk and nasal swab. However, growth of bacterial colonies showing the characteristic features of *Mycobacterium spp.* like small, moist, granular, yellowish colonies with whitish orange shine from the tissue samples (Fig. 8) that showed positivity during initial screening by smearing methods. Further colonies were analyzed by smearing methods to confirm the appearance of the acid-fast bacilli. Similar colonies of *Mycobacterium spp.* were also described by Thakur *et al.* (2010) and Konch *et al.* (2018). However, Ashenafi *et al.* (2013) and Konch *et al.* (2018) isolated *Mycobacterium spp.* from the nasal swab of tuberculin positive cattle. *Mycobacterium spp.* was earlier isolated from the lung tissue of necropsied animal (Muller *et al.* 2008, Thakur *et al.* 2010, Konch *et al.* 2018).

Detection and identification of MTB DNA in milk and

nasal swab of SICCT positive animals and tissue samples from dead animals were attempted to amplify the hsp65 and IS6110-PCR region targeting a band size of 441bp and 123bp respectively. No amplification band could be seen from the DNA of milk and nasal swab. However, amplification band for both the targeted gene could be seen from the DNA extracted from the tuberculous nodules of lung tissue (Fig. 9 and Fig. 10). As Hsp65 is mycobacterial genus specific primer and IS6110 was *Mycobacterium tuberculosis* complex specific primer, hence, amplification of both confirmed that samples were positive for tuberculosis with any of the species of MTB complex. In relation to clinical specimens the highest positive rate for lymph node and CSF specimens, was obtained by IS6110 (23.1%) and hsp65 (33.3%) genes respectively (Khosravi *et al.* 2017). Romero *et al.* (1999) reported PCR as being a more sensitive and specific test than the tuberculin test.

## CONCLUSION

This study describes isolation, and molecular identification of *Mycobacterium tuberculosis* complex (MTBC) from an organized farm of Assam. As the lesions of bovine tuberculosis are typical then a tentative diagnosis can be made by gross lesions on necropsy. Histopathological examination of the lesions confirms the diagnosis with special stain but bacteriological isolation from the lesion is the only way to make a definitive diagnosis of *Mycobacterium bovis* species which is considered as gold standard test. It has been proved that PCR targeting hsp65 and IS6110 gene if used in combination than can give rapid, accurate, sensitive and specific test to diagnose the bovine tuberculosis. This disease has zoonotic importance, and it may be transmitted to the farm worker and veterinary practitioners frequently due their occupation. So, rapid and accurate diagnosis of the disease might help in the prevention and control of bovine tuberculosis.

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